Figures and figure supplements

Stromule extension along microtubules coordinated with actin-mediated anchoring guides perinuclear chloroplast movement during innate immunity

Amutha Sampath Kumar et al
Figure 1. Chloroplast stromules extend along microtubules. (A) MTs were marked by transiently expressing TagRFP-MAP-CKL6 (yellow) in N. benthamiana transgenic plants expressing NRIP1-Cerulean that marks stromules (cyan). Confocal micrographs of stromule-to-MT interactions in lower

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epidermal pavement cells are shown. Arrows indicate stromule beaking (1), tip contact (2), extension along (3), kinking (4) and branching (5) associations with MTs. Approximately 11% of stromules were not attached to MTs (2, arrowhead). Images are maximum intensity projection of confocal z-stacks. Scale bars equal 2 μm. Total of 103 stromules were observed in 11 biological replicates to generate this data. (B) Stromules were marked by expressing NRIP1(cTP)-TagRFP in transgenic GFP-TUA6 N. benthamiana plants. Time-lapse images were acquired and kymographs over 2 min were generated. Stromules were observed extending along MTs (left) and in both directions along MTs (right). Kymographs (bottom) were generated adjacent to the red lines in top images. (C) MTs were marked by expressing EB1-Citrine (yellow) in N. benthamiana transgenic lines expressing NRIP1-Cerulean that marks stromules (cyan). Stromules were observed extending with only the tip being pulled along MTs. Kymographs (bottom) were generated adjacent to the red lines in top image and show that a stromule tip translated along a MT at a constant rate and then rapidly changed direction (arrowhead). (D) The average velocity of stromules along MTs was calculated from manually tracked stromule tips moving along MTs marked with EB1-Citrine or GFP-TUA6. Data represented as the mean standard error of the mean (SEM), ****p<0.0001 by a Student’s t-test with Welch’s correction. (E) A stromule tip was tracked using a combination of fuzzy c-means and active contour algorithm, with shape analysis to calculate the length of the stromule, the tip velocity and the association with microtubules (Lu et al., 2017). Tip associations (green dots) with MTs (gray scale) were mapped over a time series. Tips not associating with MTs are depicted as red dots. Moving stromule tips were associated with MTs except when stromules were retracting (arrowhead).

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Figure 1—figure supplement 1. Transmission electron microscopy of stromule-to-microtubule interactions. (A) Stromules were induced by transiently expressing the p50 effector for 42 hr in plants containing the N immune receptor. Serial sections were obtained and an overview montage image showing the position of stromules (S) in relation to the body of the chloroplast (Ch), mitochondrion (M), and nucleus (N). Scale bar equals 2 μm. (B) A magnified view of blue boxed area in A showing two stromules (S1, S2). (C) A further magnified area of the cyan boxed area in B shows close proximity of microtubules (gold arrow heads) along the side of a stromule (S1) and along the tip of a stromule (S2). (D, E) Two serial sections are shown to show the continuation of the close proximity of stromules to microtubules.

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Figure 2. Microtubules direct stromule movement along the ER. (A) The endoplasmic reticulum (ER) were marked by transiently expressing SP-Citrine-HDEL (magenta) and MTs were marked by TagRFP-MAP-CKL6 (yellow) in N. benthamiana transgenic lines expressing NRIP1-Cerulean that marks stromules (cyan). Confocal microscopy time-lapse images of lower epidermis of leaves indicating changes in stromule extension along MTs and push through the ER network (bottom right, merged). Arrows indicate stromule extension within an ER channel (ER) along MT. (B) High resolution airyscan confocal micrograph showing the interaction of the stromule (S) with the MT within the ER channel. (C) High-resolution time-lapse images showing the extension of stromules (cyan) along the MTs (yellow) within and away from the ER channel (magenta). Arrows indicate active stromule extension, while the ER follows the course of the extending stromule (203 s) followed by the ER remodeling (219 s) independent of the stromule extension. Scale bars equal 5 μm.
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Figure 3. Microtubule disruption leads to stromule retraction and microtubule stabilization increased stromules. (A) Dynamics of MT depolymerization and stromule retraction in the lower epidermis of NRIP1-Cerulean transgenic *N. benthamiana* plant leaves after infiltration of mock control (0.2% DMSO) or MT depolymerizing agents, APM (20 μM) or Oryzalin (300 μM). Images are maximum intensity projections represented at time points with a 5-min interval after infiltration. Arrows in mock (top row) indicate extended and branching stromules. MT depolymerization due to APM and Oryzalin causes simultaneous stromule retraction within 15 min (arrowhead; middle and bottom panels). A beaking was initiated but failed to progress to stromule (asterisk; middle and bottom panels). Scale bar equals 2 μm. (B) Stromules were increased by DMSO vehicle control treatment from 0 to 15 min compared to no significant increase from the infiltration media control. Compared to the DMSO vehicle control, stromules significantly decreased after treatment with APM (20 μM) and Oryzalin (300 μM) at 15 min and no other comparisons were significant. The experiment was repeated four times with three to five replicates per experiment. Error bars represent mean ± standard error of the mean (SEM) **p<0.05. (C) Treatment with microtubule stabilizing agent Paclitaxel (0.8 nM) produced multiple stromules from single chloroplast after 30 min (arrows, bottom left panel). The extended stromules overlapped with the MTs (arrows, merged panel). Images are maximum intensity projections. Scale bar equals 5 μm. (D) Paclitaxel treatment increased stromules per chloroplasts after 30-min treatment compared to mock treated leaves. The experiments were repeated four times with two replicates per experiment. Error bars indicate mean ± standard error of the mean (SEM) **p<0.05 by a Student’s t-test with Welch’s correction.

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Figure 4. Alterations of MT is correlated with NbGCP4 silencing. (A) In GFP-TUA6 transgenic N. benthamiana leaves that mark MTs (yellow), NbGCP4 silencing resulted in hyper-parallel and occasional bundling of MT (VIGS-NbGCP4, right) compared to the VIGS vector control (VIGS-EV, left). Images are maximum intensity projections of confocal z-stacks. Scale bar represents 40 μm. (B) qRT-PCR analysis of transcript levels of NbGCP4 in VIGS-EV control and VIGS-NbGCP4 plants showed a significant decrease in NbGCP4 transcript level 4 days after silencing by VIGS-NbGCP4 compared to the VIGS-EV control. Data represented as the mean ± standard error of the mean (SEM), n = 12, ***p<0.001 (Student’s t-test). (C) Azimuthal angles were analyzed by converting angles to mean resultant length (MRL) by converting the MT angles into individual vectors, adding the vectors together, and calculating the mean. MRL values are between 0 and 1, with 0 indicating that MT angles are random and one indicating all MT angles are the same and completely aligned. Data represented as the mean ± standard error of the mean (SEM), *p<0.01 (student’s t-test). (D) SOAX analysis was conducted on the images in (A). MT filaments are color-coded based on the azimuthal angle so that parallel MTs are the same color. (E) Curvature analysis that measures the rate of change of tangent vectors shows MTs in VIGS-NbGCP4 have less curvature. Box covers from first to third quartiles while a bar in the middle of the box indicates median. Whiskers show from minimum to maximum. ****p<0.0001 by Mann-Whitney test. (F) Analysis of the snake length computed by SOAX analysis showed an increase in MTs length in VIGS-NbGCP4. Data represented as median and 95% confidence interval. ****p<0.0001 by Mann-Whitney test.

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Figure 4—figure supplement 1. Plant morphology at different days post-silencing of NbGCP4 in NRIP1-Cerulean expressing N. benthamiana plants. (A) Plants after 4 days of virus induced gene silencing of NbGCP4 (VIGS-NbGCP4) vs. control (VIGS-EV). (B) Plants after 6 days of VIGS-NbGCP4 vs. VIGS-EV. (C) Fluorescence images of NbGCP4 expression 6 days after VIGS-NbGCP4 treatment. (D) Plants after 14 days of VIGS-NbGCP4 vs. VIGS-EV.
Figure 4—figure supplement 1 continued

NbGCP4 showed no detectable growth or morphological phenotype compared to VIGS vector (VIGS-EV) control plants. (B) Plants after 6 days of VIGS-NbGCP4 showed a growth defect and crinkled leaves compared to the VIGS-EV control plants. (C) Stromule induction and branched stromule phenotype in VIGS-NbGCP4 cells was more pronounced after 6 days of silencing compared to VIGS-EV control. However, NRIP1-Cerulean leaked out of chloroplasts and accumulated in the cytosol (arrowhead) and nucleus (arrow). (D) Plants after 14 days of NbGCP4 silencing showed growth arrest and variegated leaves. Each silencing experiment included four plants for VIGS-NbGCP4 and VIGS-EV. Experiment was repeated three times.

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Figure 5. Microtubule stabilization induces stromules constitutively. (A) Stromules were induced 24 hr after TMV-p50 effector infiltration as described in Caplan et al. (2015) (bottom left) compared to a mock control (top left). Stromules were induced both in mock (top right) and p50 infiltrated (bottom right) NbGCP4-silenced plants indicating that NbGCP4 silencing induces stromules constitutively. Stromules were often long and branched in mock-treated NbGCP4-silenced plants (top right, yellow arrowheads) compared to the VIGS-EV control (top left). Scale bar represents 40 μm. (B) Quantitative representation of stromules from A. Stromules were significantly induced in mock-treated NbGCP4-silenced plants compared to the mock-treated VIGS-EV control (compare left green bar and magenta bar). Stromules increased significantly in VIGS-EV control plants treated with p50 effector compared to the mock (compare green bars). No significant difference in stromule number was observed in NbGCP4-silenced mock-treated plants compared to the p50-treated plants (compare magenta bars). Four images per leaf were generated for quantification from total of 12 leaves for each condition. Data represented as the mean ± standard error of the mean (SEM), ***p<0.001 (Student’s t-test with Welch’s correction). (C) Stromule length was significantly increased in mock-treated VIGS-NbGCP4 plants compared to the VIGS-EV control (left open bars). p50-effector-induced immune response increased stromule length in VIGS vector control plants compared to the mock-treated VIGS-EV control plants (compare open red bars). No significant change was observed between p50-treated and mock-treated VIGS-NbGCP4 plants (compare red open bars). Box and whisker plot was drawn with rank transformation. Box cover from first to third quartiles while a bar in the middle of box indicates median. Whiskers show from 5% to 95% of ranking. ***p<0.001, ♦ comparison with VIGS-EV control, p<0.001 by Mann-Whitney test. Dots in the graph indicate outliers. (D) The velocity of stromule extension and retraction in VIGS-EV control and VIGS-NbGCP4 with or without TMV-p50-induced immune response increased stromule length in VIGS vector control plants compared to the mock-treated VIGS-EV control plants (compare open red bars). Box and whisker plot was drawn with rank transformation. Box cover from first to third quartiles while a bar in the middle of box indicates median. Whiskers show from 5% to 95% of ranking. ***p<0.001, ♦ ♦ comparison with VIGS-EV control, p<0.001 by Mann-Whitney test. Dots in the graph indicate outliers. (E) TMV-p50-induced immune response resulted in hyper-parallel MTs (NN+p50, right) compared to the control (nn+p50, left) in transgenic N. benthamiana leaves that mark MTs (yellow). Images are maximum intensity projections of confocal z-stacks. Scale bar represents 20 μm. (F) Azimuthal angle differences of MT filaments were measured by the length of the arc. Data represented as the mean ± standard error of the mean (SEM), p=0.0713
Figure 5 continued

(Student’s t test with Welch’s correction). (G) Curvature analysis that measures the rate of change of tangent vectors shows MTs in NN + p50 have less curvature. Box covers from first to third quartiles while a bar in the middle of box indicates median. Whiskers show from minimum to maximum. ******p<0.00001 (Mann-Whitney test). (H) Analysis of the snake length computed by SOAX analysis showed an increase in MTs length in NN + p50. Data represented as median and 95% confidence interval. ***p<0.001 by Mann-Whitney test.

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Figure 5—figure supplement 1. Quantitative analysis of stromule length and movements. (A) Stromule length was manually measured in ImageJ. Consistent with the independent measurement presented in Figure 5C, silencing of NbGCP4 (VIGS-NbGCP4) resulted in a higher percentage of stromules that were 3 μm or longer (pink) compared to VIGS empty vector (EV) control silenced plants. Total of 302, 162, 137, and 109 stromules were...
measured for VIGS-EV, VIGS-NbGCP4, VIGS-EV with TMV-p50, and VIGS-NbGCP4 with TMV p50, respectively. Fisher’s exact test was performed for comparison. *p<0.05 and ***p<0.0001. (B) Three types of stromule movement were manually tracked. More stable extension of stromule tips (green dot) was observed a smooth, constant motion with a linear trajectory (green line) (top row). Rapidly extending and retracting stromule tips (yellow dot) were sudden and produced an erratic trajectory (yellow line) (middle row). Stromule tips (magenta dot) moving sideways had no extension and moved tangential (magenta line) to the body of the chloroplast (bottom row). (C) Stromule movement types depicted in B were counted and the frequency of constant, smoothly extending stromules (green) was higher in NbGCP4-silenced samples. Total of 337, 186, 134, and 127 movements of stromules were recorded for VIGS-EV, VIGS-NbGCP4, VIGS-EV with TMV-p50, and VIGS-NbGCP4 with TMV p50, respectively. Chi-square test was performed to compare. *p<0.05, **p<0.001.

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Figure 6. Stromule are anchored to the actin microfilament network. (A) AFs were marked by expressing Lifeact-TagRFP (magenta) in N. benthamiana transgenic lines expressing NRIP1-Cerulean that marks stromules (cyan). Stromules were seen interacting with AFs at kink points (arrow heads). Tips were commonly seen not associated with AFs (Arrows). Stromules were occasionally seen oriented along AFs but not overlapping with AFs (*). Scale bar equals 5 μm. (B) An extended stromule with a tip in close proximity to an AF, became kinked at a point overlapping with an AF near the midpoint of the stromule, and retracted back to the kink point interacting with the AF. (C) A kymograph was created along the stromule adjacent to the 9.7 μm green line in panel B over 8 min. The stromule tip that was in close proximity to an AF and then rapidly retracts to an actin anchor. It remained attached to the actin anchor point for an additional 4 min before retraction to the body of the chloroplast. (D) The percent of stromules pausing at AFs during retraction events was quantified. No pausing resulted in a full retraction back to the body of the chloroplast (grey bar). Stromule retractions that did not retract completely and paused for multiple time frames showed a correlation of the paused stromule tip with an AF (magenta bar) or no correlation with an AF (black bar). Data was collected from 22 biological replicates spanning eight different experimental replicates. Eighty-two retraction events were quantified from 30 different cells. Data represented as the mean ± standard error of the mean (SEM), **p<0.001, ****p<0.00001 (Student’s t test with Welch’s correction). (E) AFs were marked by expressing Citrine-mTalin (magenta) in N. benthamiana transgenic lines expressing NRIP1-Cerulean that marks stromules (cyan). High-resolution airyscan confocal micrographs revealed thinning points of stromule or chloroplast interactions with AFs (arrowheads). Scale bar equals 2 μm.
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Figure 6—figure supplement 1. Chloroplast stromules statically interact with actin microfilaments. (A) AFs were marked by expressing Lifeact-TagRFP (magenta) in N. benthamiana transgenic lines expressing NRIP1-Cerulean that marks stromules (cyan). Stromules were seen in close proximity to AFs (left). Magnified image (top right) and profile lines of fluorescence intensity (bottom right) show stromules do not overlap with AFs. Images are deconvolved confocal micrographs. Scale bars equal 2 μm. (B) Three additional representative examples showing an extended stromules (top row) that partially retracted to an AF (middle row) before fully retracting (bottom row). Example 1 shows a clear kink in the stromule similar to the example in Figure 6. Examples 2 and 3 show stromules that are slightly curved that partially retract near to the location of curvature. Scale bar equals 5 μm.

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**Figure 6—figure supplement 2.** Characterization of actin microfilaments associated with stromules and the body of chloroplasts. (A) Stromules were induced by transiently expressing the TMV-p50 effector for 42 hr in plants containing the N NLR immune receptor. A transmission electron micrograph overview montage showing the position of stromules (S) in relation to the body of the chloroplast (Ch), mitochondrion (M), and nucleus (N) can be found in Figure 1—figure supplement 1. Serial sections were acquired, and in one section a stromule with a kink (K) was seen associated with an AF bundle. (B) A magnified view of the boxed area (green) in A shows the close proximity of the AF bundle (arrowheads) with a stromule kink (K). Scale bar equals 2 μm. (C) The z-stack of confocal microscopy data represented Figure 6D was rendered in the Amira software package. The AF network was skeletonized (magenta) and thick bundles were volume rendered (orange). NRIP1-Cerulean (Cyan) in the chloroplast stroma was surface rendered. (D) The surface rendering of the NRIP1-Cerulean show grooves (arrows) along the body of the chloroplasts that correspond to the location of AFs. Thinning of the stromules was also evident (arrowhead).

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Figure 6—figure supplement 3. Disruption of actin filaments does not affect stromule number. (A) AFs were marked by transiently expressing Citrine-mTalin (magenta) in N. benthamiana transgenic lines expressing NRIP1-Cerulean that marks stromules (cyan). Leaves were infiltrated with either Cytochalasin D (CTD; 200 μM) or a mock control. Arrows indicate free ends of the stromules in Mock and CTD treatments. Arrowhead indicates stromule in a loop attached to the plastid body after the treatment with CTD. Images are maximum intensity projections of deconvolved confocal z-stacks. Scale bars equal 10 μm. (B) CTD or mock (M) solution was infiltrated in leaves and imaged immediately (0–5 min) or after 30 min of treatment. There was no significant difference in stromule numbers following CTD treatment compared to mock control. The experiments were repeated more than three times. A total of 176, 208, 188, and 175 stromules from 320, 344, 310, and 297 chloroplasts were recorded for mock at 0–5 min, CTD 0–5 min, mock 30 min, and CTD 30 min after treatment respectively. Data was collected from 15 maximum intensity z-stack projections from five biological replicates for each condition. Error bars indicate mean ± standard error of the mean (SEM). ns, not significant at p=0.7943 and p=0.9894, respectively (Student’s t-test).

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Figure 7. Disruption of cytoskeleton change stromule dynamics. (A) Time lapsed images of stromules 1 hr post-treatment with 1 μM of MT inhibitor oryzalin (ORY) or with 1 μM of actin inhibitor cytochalasin D (CTD) on the leaf of the NRIP1-Cerulean transgenic N. benthamiana. At these concentrations, ORY disrupt MT organization slightly while no visible effect on actin cytoskeleton. On the other hand, CTD showed significant disruption of the actin filament while no significant effect on MT organization (see Figure 7—figure supplement 1). The experiments were repeated three times with six replicates per treatment. Scale bar equals 20 μm. (B)-(D) Quantification of stromule dynamics in A. (B) Stromules length did not change significantly upon inhibitor treatments. (C) ORY treatment increased stromule extension velocity (magenta bars compared to green bars), while CTD treatment reduced the velocity of both stromule extension and retraction (blue bars compare to green bars). Data represented as the mean ± standard error of the mean (SEM), ****p<0.0001 (Dunn’s multiple comparison test). (D) The frequency of constant, smoothly extending stromules was increased (left panel) and the frequency of sudden extending stromules decreased (right panel) with CTD treatment. ORY treatment showed no significant difference. Data represented as the mean ± standard error of the mean (SEM), *p<0.05, (Mann-Whitney test).

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Figure 7—figure supplement 1. Effects of inhibitor treatments on the disruption of cytoskeleton. *N. benthamiana* GFP-TUA6 transgenic plants were used to mark microtubule (MT, yellow) and *N. benthamiana* FABD2-GFP transgenic plants were used for actin filament (AF, magenta). Treatment with 1 μM Oryzalin (ORY) for one hour generated diffusion of GFP fluorescence and short MT fragments (top middle panel) compared to 0.1% DMSO control (top left panel). At this concentration of ORY, there was no major effect on AF compared to DMSO treatment (bottom left and middle panels). Treatment with 10 μM cytochalasin D (CTD) for 1 hr completely abolished actin cytoskeleton (bottom right panel) compared to DMSO control (bottom left panel). At this concentration of CTD, MT organization is relatively same as control (top right and left panels). All scale bars are 20 μm.

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Figure 8. Stromule directed chloroplast movement. (A) Stromules and chloroplast movement events were imaged in NRIP1-Cerulean N. benthamiana transgenic plants. A chloroplast was tracked (red line) in time lapsed images. The direction of movement was correlated with the direction of the stromule. A connected chloroplast (asterisk) moved with the stromule directed chloroplast. Time is measured in seconds and scale bars equal 2 μm. (B) AFs and stromules were marked by transiently expressing Lifeact-TagRFP (magenta) and NRIP1(cTP)-TagBFP (cyan) in N. benthamiana transgenic plants expressing GFP-TUA6 that marks MTs (yellow). The top row shows the merged images and the bottom row is an illustration highlighting the MT and actin dynamic events. At 0 min, the stromule tip is bound to a MT and a branch point is bound to an AF. At 1 min, the stromule extended along the MT. At 3 min, the stromule retracted to an actin anchor point. At 8 min, the stromule re-extended along a MT. At 9 min, the stromule retracted and correlated with chloroplast movement. Scale bar equals 5 μm. (C) The direction of the stromule connected to the chloroplast body and the direction of chloroplast movement were measured in FIJI ImageJ. The difference in angle was calculated and plotted. Both Mock and Oryzalin (ORY), showed a high frequency of values close to 0. Randomly generated values were used as a control. (D) The percent of chloroplast movement that were stromule directed movements were quantified. Cytochalasin D (CTD) treatment resulted in a complete halt of movement. Oryzalin (ORY) treatment caused a decrease in stromule directed movement compared to the DMSO vehicle control. Data represented as the ±SEM, ****p<0.0001, **p<0.01 (one-way ANOVA). (E) Stromules retract more frequently (ORY; pink bar) and extend less frequently (ORY; magenta bar) with oryzalin treatment. Data represented as the mean ± standard deviation (SD), *****p<0.00001 by Mann-Whitney test.

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Figure 8—figure supplement 1. Stromule-directed movement. (A) Stromules and chloroplast movement events were imaged in NRIP1-Cerulean N. benthamiana transgenic plants. (B) The orientation angle of the stromule connected to the chloroplast body (X-axis) and the movement of the chloroplast body (Y-axis) were plotted for the DMSO vehicle control, Oryzalin (ORY) treatment, and randomly generated control.

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Stromule-directed chloroplast movement during partial actin disruption. (A) AFs and MTs were marked by transiently expressing Citrine-mTalin (magenta) and TagRFP-MAP-CKL6 (yellow) in N. benthamiana transgenic plants expressing NRIP1-Cerulean that marks stromules (cyan). Time-lapse images of lower epidermal pavement cells of leaves indicated changes in stromule and chloroplast movement after treatment with Cytochalasin D (200 μM). Stromules were retained early (3 min) after treatment with Cytochalasin D and then briefly disrupted (8 min) with rounding of chloroplasts. Stromules re-extended (23 min) and then initiated movement of the chloroplast body (53 min). The chloroplast body moved in the direction of stromule trajectory (58 min). Arrowheads point to the chloroplast body anchored to a fragment of actin. Arrows point to stromules. Scale bar equal 10 μm. (B) Stromules and chloroplasts were tracked using the algorithm described in (Lu et al., 2017). The chloroplasts on the left in panel A had two stromules (green) resulting in opposing forces and minimal movement of the chloroplast body (red). (C) A single stromule (green) from the chloroplast on the right in panel A resulted in a rapid pulling of the chloroplast body (red). The image series in B and C span a 3-min interval of Video 7.

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Figure 9. Chloroplasts and stromules positioning during perinuclear chloroplast clustering during immune response. (A) TMV-p50 effector and Lifeact-TagRFP (magenta; AFs) were expressed in transgenic NRIP1-Cerulean N. benthamiana plants that marks stromules (cyan). Time lapse images of stromule retraction toward the nucleus (N) after chloroplast positioning around the nucleus. A stromule tip (arrow head) remained stably associated with an AF associated with a nucleus and the body of the chloroplast was anchored away from the nucleus for 18 min. Stromule retraction from 18 to 30 min brought the chloroplast body (arrow) in close association to the nucleus. The body of the chloroplast was tracked (red line). Arrows indicate retracting stromule. Scale bar equals 5 μm. (B) TMV-p50 effector and Lifeact-TagRFP (magenta; AFs) were expressed in transgenic NRIP1-Cerulean N. benthamiana plants and then fixed as described previously (Caplan et al., 2015). Three interaction points of stromules with actin surrounding nuclei were detected (circles). The body of a chloroplast was also associated with perinuclear AFs (arrow). Image is a deconvolved maximum intensity projection of a confocal microscopy z-stack. (C) Enlargements of individual xy slices of the z-stack show connections of stromule tips (left) and a stromule kink point (top right) with AFs.

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Figure 9—figure supplement 1. Stromule association with perinuclear actin microfilaments. (A) TMV-p50 effector and Citrine-mTalin (magenta; AFs) were expressed in transgenic NRIP1-Cerulean N. benthamiana plants that marks stromules (Cyan). Stromules interacted with AFs associated with nuclei. Scale bar equals 5 μm. (B) Enlargements boxed in A showing stromule tips associated with perinuclear AF. The stromule channel (Cerulean, top row) and actin channel (magenta, middle row) are shown separately and merged (bottom row). Scale bar equals 2 μm.

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Figure 10. Perinuclear chloroplast clustering during immune response. (A) Time course images of perinuclear chloroplasts during TMV-p50 effector induced immune responses compared to mock control. Chloroplasts and stromules marked by NRP1-Cerulean were pseudocolored cyan while nuclei and cytoplasm were pseudocolored red. N, nucleus. Scale bar equals 20 μm. (B) Quantification of perinuclear chloroplasts during TMV-p50 effector induced immune response compared to mock control shown in A. Ratio of nuclei associated with more than four chloroplasts in TMV-p50 infiltrated cells (magenta bars) are compared to those in control cells (green bars). More than 92 nuclei were observed for each condition from 48 images.

*Figure 10 continued on next page*
Experiments were repeated three times with four plants each. Data represented as the mean ± standard deviation (SD), **p<0.001, *p<0.01 (Student’s t test with Welch’s correction). (C) Actin cytoskeleton disruption by treatment with cytochalasin D (CTD) lead to dissociation of chloroplasts near the nucleus while microtubule disruption by treatment with oryzalin (ORY) does not affect the chloroplast positioning. Perinuclear clustering of chloroplasts was induced by TMV-pS0 effector. Images were acquired 36 hr after induction. Cytoskeleton Inhibitors were treated 35 hr after induction. Chloroplasts and stromules presented by NRIP1-Cerulean were pseudocolored cyan, while nuclei were pseudocolored red. N, nucleus. Scale bar equals 20 μm. (D) Quantification of cells associated with more than four chloroplasts in C. More than 60 nuclei were observed for each condition from 12 images. Experiments were repeated three times with two plants each (total 6 plants). Data represented as the mean ± standard deviation (SD), **p<0.001 (Student’s t test with Welch’s correction). D, DMSO; O, oryzalin; C, cytochalasin D.

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Figure 10—figure supplement 1. Perinuclear clustering of chloroplasts in TMV-p50 effector induced immune response. (A) Frequency map of nuclei associated with different number of chloroplasts in three time points after induction of immunity by TMV-p50 effector. Nucleus number with more than four chloroplasts were increased from 24 hr to 36 hr after induction (red, orange, and yellow in bars). (B) Frequency of nucleus clustered with more than four chloroplasts were decreased upon actin filament inhibitor, cytochalasin D (C), while microtubule inhibitor, Oryzalin (O) showed no difference from control (0.1% DMSO, D). DOI: https://doi.org/10.7554/eLife.23625.049